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Clinical Correlates Among 49 Families With Hemophilia A and Factor VIII Gene Inversions

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Inversions between a gene A copy within intron 22 of the factor VIII gene and additional copies outside the factor VIII gene were found in 49 families with hemophilia A. Inversion patterns were that of recombination with a distal gene A copy in 34, a proximal copy in 14, and a third (variant) copy in one. Baseline factor VIII clotting activity levels were <1% of normal in 43 and 1% in 6. No inversion was detected in 61 other families whose affected members had ≤1% activity levels nor in 42 families with moderately severe hemophilia A and 2–5% baseline levels.

Both high titer and low level alloantibody inhibitors were found in patients with or without an inversion. Of 13 high titer inhibitors, 8 were persistent and 1 of these patients had an inversion. Of 5 that responded to daily factor VIII infusions, 4 were in patients with gene inversions.

Of the 49 families with an inversion, the occurrence of hemophilia was isolated in 30 and the mother was a carrier in the 25 in which additional family members were informative. In three of these families with isolated occurrence, the maternal grandmother was a carrier whereas in three others a *de novo* mutation occurred in the maternal grandfather's factor VIII gene.

Screening for gene inversions in patients with severe (or "borderline" severe) hemophilia A provides a direct marker of the mutation in 45% of families. It is useful even if there is no living affected member and in predicting the likely severity of an infant in which there are no reliable baseline clotting activities, including 70% of families with isolated occurrences of hemophilia A. © 1996 Wiley-Liss, Inc.

Key words: hemophilia A, factor VIII, mutations

INTRODUCTION

Factor VIII deficiency in severe hemophilia A frequently results from intrachromosomal homologous recombination (cross-over) between a "gene A" copy located in factor VIII's intron 22 and 1 of 2 additional copies that are 500 kb toward the telomer, Xq-ter [1,2]. This rearrangement dislocates and inverts the first 22 (of 26) exons of the factor VIII gene, preventing formation of a full length transcript and leading to severe hemophilia A without detectable circulating factor VIII protein [3]. The inversions are recognized by distinctive patterns of BclI-digested genomic DNA on Southern blots, hybridized with gene A probe. A 21.5 kb band corresponds to the intron 22 gene A copy and 16 kb and 14 kb bands correspond to the more distal and proximal copies, respectively [1,3]. Occasionally, variant patterns are found, some of which appear due to a third gene A copy that is also outside of the factor VIII gene [1]. Of several series reported [4–19], there is remarkable consistency with 40 to 45% of families with severe hemophilia A having a gene inversion. Recombination between the intron 22 and more distal gene A copy occurs most frequently; in most series, recombination with the proximal copy is distinctly less frequent.

In families in which the affected member had an inversion but there was no prior family history (isolated hemophilia A), 49 of 50 mothers carried the inversion [21]; furthermore, haplotype analysis allowed assignment of origin to a maternal grandparent in 23 families and each

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inversion originated in the maternal grandfather's factor VIII gene [21,22]. These family studies are best explained by the absence of a homolog of Xq to pair with and inhibit recombination. No inversions were found in analysis of 79 normal factor VIII genes [5]. However in one case, the intensity of bands in a carrier mother suggests somatic mosaicism [23]. In using Southern blot analysis for carrier testing, an unusual pattern was encountered by Peretz et al. [9] that appears to have been due to a second cross-over event, in oogenesis, that could falsely assign carrier status to a woman whose Xq28 region contains both an intact factor VIII gene and the exon 1–22 portion of an inverted, extragenic sequence [9].

Among patients with inversions, clinical correlates have been more variable and less well specified in published series. Considering the clinical severity of the bleeding tendency, most series have only screened patients with clinically severe hemophilia A. Whereas manifestations of a "typical" patient with a severe bleeding tendency are widely accepted, clinical overlap with moderately severe hemophilia A has not been examined. Of 72 Spanish patients with moderately severe or mild hemophilia A, however, none showed the inversion [13]; in the only other series including moderately severe hemophilia A patients, two of ten carried the deletion [17]. In the latter report, however, the degree of clinical severity was only known in one third of the referred hemophilia A patient samples. A second issue is inhibitor development. In one series, inhibitors were not found in any of 11 patients with inversions compared to 7 of the 12 severe hemophilia A patients without gene inversions [6]. In other series, however, patients that develop alloantibodies were among those with a factor VIII gene inversion [5,7,8,11–17].

The current series examines clinical severity and inhibitor development in severe and moderate to severe hemophilia A patients and their families. This series is distinctive in that 1) 29% of families with a factor VIII gene inversion have cross-over events that occurred between factor VIII and the proximal gene A copy, 2) 12% of the inversions occurred in patients whose baseline factor VIII levels had been reported as 1% of normal, and 3) patients with high titer inhibitors that were transient or that developed tolerance were predominantly among those with inversions.

MATERIALS AND METHODS

Hemophilia A Patients

Patients and/or family members from 152 families were examined for a factor VIII gene inversion by Southern blots. All but three of these families have affected members that have been followed regionally in the Pacific Northwest. Familial hemophilia is defined as an X-linked bleeding disorder being present in a patient or obligate carrier for at least two generations prior to the propositus (index case); isolated hemophilia is where the patient is the only known affected member or where a second affected brother or nephew was subsequently born. As several of the families have been followed up to 22 years, the propositus was deceased in some such that samples on other affected members or obligate carriers were studied. Samples were obtained either with informed consent by a protocol approved by the University of Washington's Human Subjects Review Committee or as submitted by families for carrier testing.

An "arthropathy index" was adapted from a functional rating questionnaire used in the Netherlands [24]. A value of 0 to 3 was summed for right and left elbows, knees, ankles, and either shoulder or hip joints. Based upon joint measurements during periodic assessments, 0 is no impairment, 1, loss of $<10°$ at either extreme of flexion or extension (or 10% for ankles), 2, 10–20° (or %), and 3, $>20°$ (or %) or status post joint fusion or replacement.

In 99 of 152 families studied, baseline factor VIII levels were $<1\%$ and all of the affected members had a clinical diagnosis of severe hemophilia A, based upon frequent, spontaneous joint bleeding and the use of several transfusions per year to treat acute bleeding episodes. Eleven patients had other mutations and 10 of these were among 14 previously described [25,26]; the 11th (who was adopted) lacked a MaeIII restriction site in a fragment amplified from the 5′ end of his 14th exon [25], confirmed on sequencing as a C to T transition specifying an Arg^{795} to Stop codon; he is clinically severe with $<1\%$ baseline factor VIII clotting activity. In 11 of the families, the lowest baseline factor VIII level obtained in an affected member was 1%; because inversions were found among these families, they are pooled with the 99 with $<1\%$. For 42 additional patients, hemophilia A was clinically characterized as moderately severe, rarely with spontaneous bleeding episodes (except in some patients with advanced arthropathy secondary to traumatic bleeding episodes); baseline factor VIII clotting activities in these patients were 2 to 5% of normal. Where more than one value of factor VIII activity was available in the same or related hemophilic patients, the lowest reported value was taken, assuming that higher levels likely reflected residual factor VIII from recent transfusions.

Factor VIII Assays

Baseline factor VIII clotting activities had been performed on most patients through the Blood Center's Reference Coagulation Laboratory during the past 22 years. For some patients, however, baseline levels were as reported from regional laboratories; all were based on several dilutions in one-stage factor VIII clotting assays using deficient plasma as substrate [27]. For inhibitor assays, standard Bethesda (2 hr) preincubations with normal plasma were used [28]. For patients that had detectable

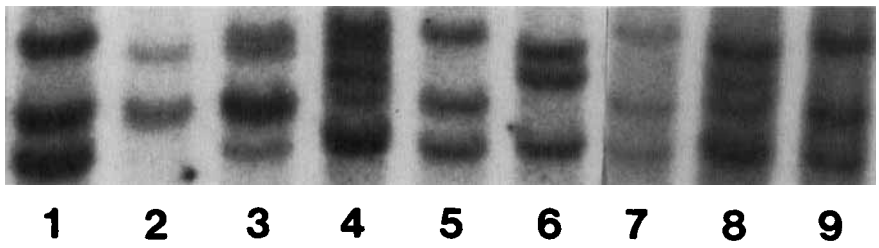

Fig. 1. Factor VIII gene inversion patterns. BclI digested, genomic DNA samples were electrophoresed, blotted onto membranes, hybridized with a ^{32}P-labeled, 0.9 kb probe, and radioautographed as described under methods. Samples were as follows: lanes 1, 5, 7, and 9, normal subjects; lanes 2 and 3, hemizygous and heterozygous (respectively) members of a family with "proximal" factor VIII gene inversion; lanes 4 and 6, heterozygous and hemizygous (respectively) members of a family with a "distal" factor VIII gene inversion. Lane 8 was DNA from a patient with a variant pattern, likely due to recombination between intron 22 "gene A" and an additional extragenic "gene A" copy.

inhibitors, titers as well as infusion response and clinical data was available over time (in terms of boost response and results of daily infusions for immune "tolerance" induction when tried). Furthermore, low initial recoveries and shortened survivals were observed in patients identified with low level inhibitors (<5 Bethesda units or BU/ml) but these did not preclude clinical responses to higher doses of factor VIII.

DNA Samples and Southern Blotting

Genomic DNA was extracted from leukocytes in EDTA-anticoagulated whole blood samples within 24 h of drawing as described [29]. BclI (New England Biolabs, Boston, MA) was used according to manufacturer's directions with overnight digests of 5–10 μg genomic DNA at 50°C followed by 3 hr of a second digest with additional enzyme. An EcoRI/SstI digested 0.9 kg fragment of plasmid p482.6 (catalog No. 57203, American Type Culture Collection, Rockville, MD), i.e., "gene A" probe [1], was ^{32}P-labeled by random primers and hybridized to nylon membranes with electrophoresed, blotted samples and radioautographed as described [30]. For all families in which an inversion was found, it was demonstrated on at least two separately digested and electrophoresed samples on either more than one affected member (including carriers) or as a repeat digest from DNA of the propositus.

Factor VIII Polymorphisms

Fragments were amplified by polymerase chain reaction for detecting BclI [31], intron 7 G/A polymorphism by amplification mismatch for AlwNI [32] and introns 13 [33] and 22 [34] fragments for CA repeat polymorphisms. The latter were amplified with ^{32}P-labeled dNTPs, electrophoresed and radioautographed.

RESULTS

Inversion Screening

In individuals from 49 families, a gene inversion pattern was seen on Southern blots and representative radioautographic patterns are shown in Figure 1. In 14 of the 49 families (29%), the pattern was of recombination with the proximal gene A copy (Table I). One patient had retained normal-sized distal and proximal fragments but had a smaller exon 22 fragment (19 kb) and a new band at 17 kb (Fig 1, lane 8). The latter is consistent with recombination of factor VIII and an additional gene A copy [1,21].

Southern blots were normal in 10 of 11 patients with previously established mutations. The intron 22 gene A copy was missing in the eleventh patient who has a previously described deletion of exon 16 through much of intron 22 including the polymorphic XbaI site [25] and the CA repeat region. Fifty additional patients of similar severity and 42 patients that were more moderately severe lacked the inversion.

Clinical Severity

Of 99 patients with severe hemophilia A and <1% factor VIII clotting activity, 43 had an inversion. Baseline factor VIII clotting activity levels were 1% in six patients with an inversion pattern (five with distal and one with proximal recombinations), one with a previously described mutation (Arg^{2163} to Cys [26]) and four of the 50 that have not yet had a mutation detected. Treatment records were available on 12 affected individuals from 10 of these families with 1% levels (Table II) and each received comparable treatment to other known severe hemophilia A patients with factor VIII levels of <1% (not shown). In 1 of the 6 with inversions (patient E), the 1% level is most likely due to a transfusion 48 hr before and in 4 others, recent transfusion remains a plausible explanation for the 1% level. Overall, 49 of 110 (45%) of those unrelated patients with factor VIII levels ≤1% had a factor VIII gene inversion.

Inhibitor Formation

Seven of the forty-nine patients with gene inversions have developed alloantibodies to human factor VIII fol-

TABLE I. Inversion Screening in Hemophilia A Patients

Result	N	N with VIII:C = 1%	Inheritance			Inhibitor[a]	
			Isolated	Familial	Adopted	≤5 BU per ml	>5 BU(tr) per ml
Inversion present							
Distal	34	5	22	11	1	2	3(2)
Proximal	14	1	7	7	—	—	2(2)
Variant	1	—	1	—	—	—	—
Subtotal	49	6	30	18	1	2	5(4)
Other mutations							
Gross Deletion	2	—	2	—	—	—	1(0)
Arg to Stop	8	—	3	4	1	2	1(1)
Arg to Cys	1	1	1	—	—	—	—
Subtotal	11	1	6	4	1	2	2(1)
Negative screening	50[b]	4	38	11	1	2	6(0)
Total	110	11	74	33	3	6	13(5)

[a]Expressed as Bethesda units (BU)/ml [28]; patients with transient (tr) high titer inhibitors are indicated in parentheses.

[b]In addition, 42 other patients with moderately severe hemophilia A and 2 to 5% baseline factor VIII clotting activities were screened and did not have a gene inversion pattern.

TABLE II. Treatment Summary for Hemophilia A Patients With 1% Factor VIII Levels[a]

Patient	Age[b] (years)	Weight[b] (kg)	Arthropathy index	Orthopedic procedures	Factor VIII units transfused	
					U/kg/year[b] (N, years)	Average N of infusions/year
Inversion present						
A_1[c]	19	60	1	0	3016 (2)	121
A_2[c]	13	44	1	1	4307 (1)	158
B	14	57	3	0	2902 (2)	110
C	14	42	4	0	1683 (2)	47
D	37	63	18	1	327 (4)	14
E	4	12	0	0	573 (2)	21
No inversion present						
F	15	66	0	0	2020 (4)	86
G	15	51	3	0	173 (2)	8
H	28	70	16	0	976 (2)	45
I_1[c]	47	83	13	0	437 (4)	20
I_2[c]	44	86	9	1	1303 (2)	56
J	4	15	0	0	188 (1)	9

[a]All patients except J are on home therapy and have had infrequent, spontaneous hemarthroses; patients E had 2 by 18 months and his latest level was 1% 48 hr after infusion. Patients E and F have had periods of prophylaxis for recurrent bleeding in a single joint that are not included in the transfusion periods summarized. Patient D avoids factor VIII infusions except for the most painful bleeding episodes. Patient G had 12 episodes one year, 4 the next. Patient I_1 had 1 year with only two episodes. "Arthropathy index" is defined in methods by grading up to eight joints on a 0 to 3 scale of functional severity for each (total range is 0–24) where 0 is normal function. Orthopedic procedures include patient A_2, knee synovectomy at age 11; patient D, knee fusion at age 15; patient I_2, ankle fusion at age 32.

[b]Average age and weight during period which treatments were examined (expressed as number of years, the value in parentheses after factor VIII units per kg/year).

[c]Families A and I each have two brothers followed.

lowing infusions. In two of these the titer has remained low (1 BU/ml) without boost response despite major surgeries. The other five patients with an inversion had high titer, boost-responding inhibitors. Inversions involving the distal and proximal gene A copies were found in three and two of these patients, respectively. In four of these five, a brother, cousin, or nephew, has severe hemophilia A without an inhibitor. In four of these patients, the high titer inhibitors have either disappeared or reverted to a low titer, non-boost responding inhibitor over time and independent of HIV status. Three of these responded to daily factor VIII infusions ("immune tolerance induc-

tion") and the fourth disappeared "spontaneously" after factor VIII treatment for a bleeding episode when the titer was low. The fifth has persisted with boost responses to >1,000 BU/ml for 17 years and he has not wanted to try daily infusions.

Of the 11 patients with other known mutations, two have had low titer, non-boost responding inhibitors, a third (with Arg^{1941} to Stop) had a high titer inhibitor that is no longer present and a fourth, with deletion of exons 15 through intron 22 [25], has a high titer inhibitor that has been resistant to attempts at immune tolerance induction.

Of the 50 additional patients with hemophilia A and factor VIII levels ≤1%, 8 developed inhibitors and two of these have been low level, non-boost responding. Of the remaining six that are high titer and boost-responding, three have been present for at least 15 years and none of the other three have responded to prolonged attempts at immune tolerance induction. Two of these six patients with high titer inhibitors have had an affected relative; in one, his uncle died with a similar, persistent inhibitor and in the another, his hemophilic brother has no inhibitor.

Inheritance

Among the 49 patients with factor VIII gene inversions, 18 were familial including 11 with a distal and 7 with a proximal pattern. One patient with a distal gene inversion was adopted. Of the remaining 30, hemophilia A was isolated and in 25 of these, DNA was available from additional family members. Of these 25 families, the same factor VIII gene inversion was present in the mother in 24 and in a sister of the 25th whose mother is deceased. In eight of these families, the obligate carrier status of the propositus's mother was established by birth of a second affected family member. Of the families examined further, DNA analyses were informative in five maternal grandparents and in a maternal aunt in a 6th. In three of these, hemophilia was present in the maternal grandmother (in one because a maternal aunt carried the mutation) whereas in three others, the grandparents lacked the mutation and intragenic haplotype analysis demonstrated that the mutation had occurred *de novo* in the patient's maternal grandfather's factor VIII gene.

For the 11 patients with other known mutations, four (all with Arg to Stop mutations) were familial, one was adopted and the other 6 were isolated (two were *de novo*, sporadic) [25]. Of the 50 additional patients with similar severity, only 11 were familial and one was adopted; the other 39 were isolated and of these, two had occurred in the maternal grandfather's factor VIII gene (by haplotype analysis). Of the 42 additional individuals with moderately severe hemophilia A, 12 were familial, one was adopted and 29 were isolated. Thus in 148 families where a family history was available, it was negative prior to the index case in 70%.

DISCUSSION

Among several large series, 40 to 45% of patients with severe hemophilia A have a factor VIII gene inversion due to homologous recombination between gene A copies [3; Table III]. Severity, however, represents a somewhat arbitrary categorization both from clinical criteria and laboratory assay results as will be discussed below. Despite a similar percentage of patients with factor VIII gene inversions, the present series differs from most others in its higher frequency of recombination with the proximal of the extragenic gene A copies, being almost twice as frequent as the average of other reports (Table III). No clear reason for the marked variability of proximal versus distal recombinations between series of different families is apparent. "Founder effects" are unlikely because the hemophilia is severe and a variety of haplotypes are found among familial occurrences (unpublished data). Of variant patterns, the one observed in the current series is similar to a "type 3" variant [1,21] and this pattern is most consistent with recombination between the factor VIII and a third extragenic gene A copy in the cluster around 500 kb from the factor VIII gene. Additional copies have been found as rare variants in normal individuals [1], and three other variant patterns have been described by Windsor et al. [11].

It is now clear that patients with factor VIII inversions can develop high titer, boost-responding alloantibodies against transfused factor VIII. Furthermore, the occurrence of these inhibitors (as well as low level alloantibodies) is essentially as frequent as in severe hemophilia A patients without factor VIII gene inversions. It is curious that of six patients with high titer inhibitors that responded to immune tolerance induction or disappeared spontaneously; four were associated with a factor VIII gene inversion; the other two have Arg to Stop codons. Only one of the eight others that persisted is in a patient with an inversion. To determine whether or not this trend (of high titer inhibitors in patients with inversions being more transient) is reflective of the population of hemophilic patients with inhibitors, it will be necessary to have more detail on inhibitor types and their persistence from patients in other series.

Traditionally, severe hemophilia A is defined at <1% of the factor VIII clotting activity when compared to the level in a pool of normal donors' plasmas. Among severe patients (<1% levels), there is some variability in terms of the frequency of bleeding episodes and the development of chronic arthropathy even within families. The concomitant inheritance of a common thrombotic risk factor such as activated protein C resistance [35] might account for some clinical variability. Although no dominant effect of this has been found among hemophilic patients, a quantitative difference remains possible [36]. Some recent assay systems, especially with automated

TABLE III. Factor VIII Gene Inversions in Severe Hemophilia A: Published and Reported Series

Series	Sev HA	Inv	% Inv	Distal	Proximal	Variant	% Proximal	Reference
A	19	9(9)[a]	47	8 (5)[a]	1 (3)[a]	(1)[a]	11	Lakich et al. [1]
B	24	10	42	7	2		22	Naylor et al. [2,4]
C	164	69	42	62	7		11	Millar et al. [5]
D	23	11	48	10	1		9	Goodeve et al. [6]
E	41	20	49	15	4	1	20	Ljung [7]
F	102	42	41	31	11		26	Tizzano et al. [8]
G	54	27	50					Peretz et al. [9]
H	85	40	47	32	8		20	Jenkins et al. [10]
I	71	32	45	23	5	4	11	Windsor et al. [11]
J	49	24	49	23	1		4	Figueiredo et al. [12]
K	27	12	44	11	1		8	Van de Water et al. [13]
L	21	12	57	7	5		42	Arruda et al. [14]
M	77	29	38	27	2		7	Inaba et al. [15]
N	71	35	49	28	7		20	Acquila et al. [16]
O	(166)[b]	(57)[b]	(34)[b]	45	10	2	18	Vnencak-Jones et al. [17]
P	27	12	44	9	2	1	17	Poon et al. [18]
Q	63	19	30	15	3	1	16	Enayat et al. [19]
R	110	49	45	34	14	1	29	This study
TOTAL	1,028	452	43	387	84	10	18	

[a]Data in parentheses are those also reported for nine of ten patients studied from an earlier series in whom no exonic mutations were detected by density gradient gel electrophoresis (Higuchi et al. [20]) and not included in totals as they are likely included in series O [17].

[b]Not included in totals as severity was only known in 59 of these families. Inversions were noted in 36 of whom were severe, 2 of 10 moderately severe and none of 13 mild hemophilia A patients.

instruments, are inaccurate at levels below 2% of normal, precluding a distinction between severe and moderately severe hemophilia A. Clotting activities may be spuriously high if there is clotting before the anticoagulant is mixed or in cord blood samples. Although transfused factor VIII has a half-life of 10 hr, it would take about 2.5 days to return to <1% from a single, routine infusion. Thus, recent infusions can account for at least some of the assay variability. It is noteworthy that only one patient with a 1% level and an inversion (Table II, patient D) had his sample drawn at a time that he clearly had not transfused for several weeks. Clinically, he is severe. Thus patients that have detectable baseline levels of around 1% of normal but with spontaneous bleeding episodes and clinical severe disorders may exist but are unusual. However, it is useful to screen patients otherwise thought of as moderately severe to severe that may have baseline factor VIII clotting activities of 1% of normal as shown in this series.

It is clear that screening for a factor VIII gene inversion in severe hemophilia A patients is worthwhile, allowing direct diagnosis and carrier testing among family members. A more rapid screening strategy, such as the reverse-transcriptase PCR method that Antonarakis et al. reported [37] will be useful (once details are published) as a method that is less labor-intensive than Southern blotting. Two advantages of direct screening for the inversion are the ability to examine samples from women in families where there is no living member and to discriminate the probable severity of newborns in isolated occurrences in families, particularly where no reliable baseline factor VIII clotting activity level is available.

As in other series, mothers of patients with factor VIII gene inversions are nearly always carriers, even in isolated occurrences. Consideration should be given to the possibility of somatic mosaicism [23] or a second recombination between the inverted segment and the normal factor VIII gene on the other chromosome during meiosis [9]. When origin in a maternal grandparent's factor VIII gene can be assigned by haplotype analysis, essentially all have originated in spermatogenesis. Thus a second son in a family with isolated occurrence of a factor VIII gene inversion is essentially at 50% risk of having hemophilia and DNA screening of a prenatal sample should be considered. Sisters have a 50% risk of being carriers. Because sporadic inversions could either originate during maternal grandfather's spermatogenesis or an earlier event that is carried "silently" by the patient's maternal grandmother, screening for carriers can be targeted to those at risk starting with the maternal grandmother, if she is available.

In Sweden, haplotype analysis of a random sample of families with isolated, severe hemophilia A, suggested that at least 55% of mutations were sporadic occurrences within the past two generations [38]. In the current series, there was no family history of hemophilia in 70% of

families whose affected members had severe and moderately severe hemophilia A. Thus between 55 and 70% of severe hemophilia A is due to a mutation within two or three generations of the propositus.

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